Transformation of *Solanum lycopersicum* (tomatoes) with a growth regulating protein apyrase

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Dedication

To my parents, for their continual support these past four years which made this thesis possible. I couldn't have done it without the life skills you both taught me.

To my sister, Grace, thanks for always reminding me to reach past what I can achieve.

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I. Abstract

In previous studies the role of apyrase in plant growth and development has been investigated in the model plant, *Arabidopsis thaliana*. Results obtained in *Arabidopsis* have suggested that ectoapyrases regulate plant growth and participate in wound responses. To apply these findings to an agriculturally relevant plant we transformed apyrase into tomatoes (*Solanum lycopersicum*, MicroTom). Our goal is to overexpress and knockdown this gene in transgenic tomato plants to determine if their function in tomatoes is similar to those found in *Arabidopsis*. We first began by constructing vectors for apyrase to either overexpress the gene through a 35-S promoter in a pCambia 2300 plasmid or knockdown the gene by RNAi. These constructs were used for *Agrobacterium* mediated transformation of tomato plants, where kanamycin was used for plant selection. Thus far we have successfully produced transgenic tomato lines with *Solanum* apyrase overexpressed as judged by RT-PCR analysis. These transgenic tomato lines will be tested for growth phenotypes including increased fruit size.

II. Background

Motivation

Ectoapyrases have been characterized as Nucleoside Triphosphate Diphosphohydrolases (NTPDase) which cleave the γ and β phosphate from adenosine triphosphate (ATP) and adenosine diphosphate (ADP), respectively (Komoszynski and Wojtczak, 1996). This catalytic ability has been linked to a role in extracellular ATP (eATP) signaling in animals and plants.

In plants, in particular *Arabidopsis thaliana*, eATP signaling is initiated by an increase in eATP concentration in the extracellular matrix. Growing root hairs release eATP during polar growth as the tip of the root expands (Kim *et al.*, 2006). In turn ectoapyrases are essential in regulating this eATP concentration so that during growth phases, eATP will not build up to inhibitory levels that signal growth inhibition. Chemical and genetic approaches have been used to demonstrate the growth regulatory function of apyrases (Wu *et al.*, 2007). For example, ectoapyrase inhibitors have been found to inhibit pollen tube growth (Wu *et al.*, 2007) and root hair growth (Clark *et al.*, ms. in review). It has been theorized that when ectoapyrase inhibitors are applied, the buildup in eATP concentration is caused by the inactivation of the NTPDase activity of apyrase. Furthermore, when high concentrations of non-hydrolyzable forms of ATP and ADP are exogenously applied to root hairs, significant inhibition of growth is seen (Clark *et al.*, ms. in review).

In addition to chemical suppression of pollen and root hair growth through inhibitors and ATP/ADP analogs, the knockdown of the expression of *AtAPY1* and

AtAPY2 (two members of the *Arabidopsis* apyrase family) resulted in dwarf phenotypes in transgenic plants due to suppressed root and shoot growth (Wu *et al.*, 2007). Moreover, overexpression of apyrase in *Arabidopsis* shows promotion of pollen tube and hypocotyl growth in early plant development (Wu *et al.*, 2007). Recent studies in the Roux lab have also indicated the potential role of apyrase in stomata opening and closure (Clark, Fraley *et al.*, manuscript in preparation).

The growth-regulation function of apyrases has also been studied in a variety of other plants. The knockdown of apyrase expression in potato results in reduced tuber size (Riewe *et al.*,2008). Enhanced apyrase expression in cotton fibers is correlated with enhanced growth of the fiber (Clark *et al.*, 2010). Finally, in a recent rice genetic screen of a root hairless mutant, *rth1*, the disrupted gene causing the phenotype was found to be apyrase (Yuo *et al.*, 2009). These findings further support the importance apyrase has in plant growth and development.

Rationale

With increasing evidence of the role ectoapyrases have in regulating vegetative growth in a variety of plants though extracellular ATP (eATP) signaling, we proposed that a further investigation into the function of ectoapyrases might reveal a similar role in controlling fruit growth. To test this we decided to investigate the role of *Sl*APY1 in tomatoes.

Due to promising discoveries from genetic studies on the growth regulating function of ectoapyrases in *Arabidopsis*, we wished to transform an agriculturally

relevant plant, tomato, to overexpress and knockdown *Sl*APY1, a tomato apyrase similar in sequence to the Arabidopsis ectoapyrases. We hoped the growth changes observed in the transformed *Arabidopsis* from Wu *et al.* (2007) could be mimicked in transformed tomatoes; we were especially interested in potential effects on fruit size. Our approach for the project was to construct two vectors that overexpress and knockdown *Solanum* (tomato) apyrase, *Sl*APY1.

Agrobacterium Transformation

Agrobacterium mediated transformation is commonly used in plant biology labs to insert new genes into plant cells for integration into the plant's genome. This process was developed based on the natural ability of a soil bacterium, *Agrobacterium tumefaciens*, to transfer its own genetic information into a host plant. In nature, *Agrobacterium tumefaciens* utilizes this genetic engineering ability to confer genetic information to form tumors at plant wound sites (crown gall). This natural exchange of genes was discovered to have laboratory relevance when the original T-DNA containing the tumor formation genes were removed and replaced with foreign DNA. This new method for plant transformation has revolutionized plant biology and has been used to produce transgenic plants ranging from monocots, dicots, conifers, marine algae, and fungi. (Cambia, 2010; Federal Ministry of Education and Research, 2006)

Micro-Tom Tomatoes

This cultivar of tomato was chosen due to its small plant size (grows up to 5-8 inches tall) and short life cycle of which produces mature fruit in 70-90 days after sowing. Due to these characteristics Meissner *et. al.*(1997) proposed using Micro-Tom tomatoes as the model system for testing tomato genetics. This miniature tomato was originally bred for urban gardens (Scott & Harbaugh, 1989) but due to its small stature it was ideal for laboratory settings which needed plants to grow at a high density in limited space. Moreover Micro-Tom tomatoes have been assessed to undergo *Agrobacterium* mediated transformation of cotyledons at frequencies of up to 80% and only differ from standard tomato cultivars by two major genes (Meissner *et. al*., 1997). The only major drawback in using Micro-Tom tomatoes is the absence of *in vitro* regeneration ability which can be corrected by transferring the high organogenetic competence from the MsK genotype to Micro-Tom plants (Lima *et. al.*, 2004).

III. Materials and Methods

Plant Material and Growth Conditions

Micro-Tom seeds were purchased through Tomato Growers Supply Company with roughly 30 seeds per packet, #6536. Seeds were first surface sterilized in 50% (v/v) commercial bleach plus 0.1% Tween-20 for 20 minutes and then rinsed with autoclaved water five to seven times (outlined from Lima *et. al.*, 2004). The sterilized seeds were allowed to vernalize in 4°C for at least three days. Prepared seeds were then planted in 325 mL pots filled with sterilized soil. Potted seeds were placed in a growth chamber at roughly 70°C under 24 hour light, watered daily.

Transgenic tomato lines were obtained from Dr. Jean Gould (Texas A&M University) after transformation. Received plants were grown in sterile containers under 24 hour light at 70°C until the callus began to exhibit plant like phenotypes (leaves and roots). Once the plants had a hypocotyl length of roughly 1.5 inches they were transplanted into 325 mL pots with sterilized soil. Plants were watered daily.

Cloning

Overview

Our approach for the project was to construct two vectors – one that overexpress and one that knocks down *Solanum* (tomato) apyrase, *Sl*APY1. Our cloning strategy was to first amplify the *Sl*APY1 gene from a Micro-Tom leaf cDNA library, which we generated from a Micro-Tom leaf tissue collection after RNA extraction and reverse

transcription. The resulting *Sl*APY1 gene is roughly 1.3 kb (Supplementary Fig. 2) and was extracted from a DNA gel. The PCR product was next inserted into a vector and transformed into *E. coli*. This process was repeated several times – Topo8/pENTR-D/TOPO vector for sequencing, pRT100 vector for the Cauliflower Mosaic Virus (CaMV) 35S promoter and polyA tail, pCambia 2300 for tomato transformation, and pB7GWIWG2(I) for RNAi knockdown. Completed constructs were sent to Dr. Gould's lab at Texas A&M University for transformation into Micro-Tom tomatoes. Transgenic tomatoes were returned to the Roux lab for screening and further experimentation.

Formation of first strand cDNA library

Eighty to one hundred mg of tissue from Micro-Tom tomatoes were needed for each library. Collected plant tissues were immediately frozen in liquid nitrogen after removal and were kept in liquid nitrogen throughout the steps leading up to RNA extraction. Tissues were broken down by a motorized hand-held pestle to a fine powder which usually took 15-20 minutes. Once broken down the powdered plant tissue underwent RNA extraction using a Qiagen RNeasy Plant Minikit. The concentration of RNA was measured using a Nanodrop and was either immediately stored in -40 °C or used for first strand cDNA synthesis. Extra care was made throughout the RNA extraction process to reduce contamination from RNase. For first strand cDNA synthesis 1 μg of RNA was used. A DNase reaction was performed to remove any DNA contaminants; the Invitrogen DNase protocol was used. Finally the remaining steps of first strand synthesis were carried out following the Invitrogen Superscript RT III procedure. The synthesized cDNA was then stored in 4°C for future use.

High fidelity PCR

Due to a low error rate, Phusion High-Fidelity DNA polymerase from New England Biolabs was used. PCR was carried out following the protocol set out in the NEB Phusion webpage with an annealing temperature of 50°C and an extension time of 45 seconds for amplifying *Sl*APY1. Primers used for amplifying *Sl*APY1 for overexpression and RNAi vectors are outlined in Supplementary Fig. 4b and Supplementary Fig. 4d, respectively. The PCR product was run on an agarose (1%) gel electrophoresis with 1X ethidium bromide (EtBr) to visualize the DNA band. The correct size band was excised from the gel and purified using a Qiagen Qiaex II Gel Extraction Kit to produce 30 μL of product. The purified PCR product was then stored at 4° C.

Sequencing

Purified PCR products used for cloning were first sequenced to assess fidelity. To sequence the insert, the product was either first cloned into the Invitrogen pENTR-D/TOPO vector (RNAi strategy) or the TOPO 8 vector (overexpressor strategy). Instructions set out in the TOPO vector manuals were used for the TOPO cloning reactions and the transformation of One Shot TOP10 Chemically Competent *E. coli*. Successful insertion into the TOPO vector was assessed by collecting plasmids from individual colonies with the Qiagen Qiaprep Spin miniPrep Kit. Plasmids were screened for the insert by performing a restriction enzyme digest with the appropriate restriction enzyme(s). If the insert was present, the plasmid was sequenced by the ICMB core facility with M13 forward and reverse primers.

Restriction Enzyme Digestion and Ligation

Restriction Enzymes (RE) used, purchased from New England Biolabs, included ApaI, KpnI, EcoRI, and HindIII. The restriction enzymes were added to a solution designated by NEB recommendations with the RE at a 5X concentration. The digest lasted for four hours and then was terminated by subsequent chemical or heat inactivation. The digested DNA was then run on an agarose (1%) gel electrophoresis with 1X EtBr to visualize the DNA bands. If the digest was successful the band was excised and purified by the same means as designated above. Digested DNA fragments, one digested with Calf Intestinal Alkaline Phosphatase (CIP) and another without CIP, were ligated together with NEB T4 DNA ligase overnight at 14 °C (roughly 18 hours).

Vectors

pRT100

The pRT100 vector (Supplementary Fig. 5a) was used in the overexpressor strategy to attach the CaMV 35S promoter and polyA tail to our *Sl*APY1 gene. The *Sl*APY1 gene was first amplified using primers designed to add an ApaI RE cut site on the 5' end and KpnI RE cut site on the 3' end (Supplementary Fig. 4b). These RE sites were added so that *Sl*APY1 could be directionally cloned into pRT100. Once the gene was amplified with the appropriate cut sites it was ligated into TOPO8 for sequencing. When the gene sequence was confirmed to be correct, the TOPO8 *Sl*APY1 vector was subjected to a digestion with ApaI and KpnI. Concurrently, a digestion of pRT100 was carried out with the same restriction enzymes and CIP, which prevented any re-ligation of the pRT100 vector. Both digests were separated by gel electrophoresis and the *Sl*APY1

(1.3kb) band and the pRT100 (3.34kb) band were excised and purified. The purified insert (*Sl*APY1) and the purified pRT100 were then ligated together overnight and then transformed into E. coli. Colonies were screened until successful constructs of *Sl*APY1 and pRT100 were found. These constructs were then sent for sequencing using designed primers for the promoter and terminator regions (Supplementary Fig. 4c).

pCAMBIA 2300

The pCAMBIA 2300 vector (Supplementary Fig. 5b) was chosen because of previous literature using pCAMBIA vectors in tomato transformations as a minimal selection vector. Additionally, we chose the 2300 vector due to its kanamycin plant selection which is easily used for screenings. The pRT100 construct with *Sl*APY1 was first digested with Hind III along with the pCAMBIA 2300 vector with CIP added. The digested fragments were run on a DNA gel from which the *Sl*APY1 segment (2.0 kb) band and the pCAMBIA 2300 (8.742 kb) band was excised and purified. The purified fragments were then ligated together to form our final construct. Successful ligations were screened for after subsequent transformations of *E. coli*, plasmid isolations, and restriction digests. Our final construct of pCAMBIA 2300 with *Sl*APY1 and associated promoter/terminator regions was then screened for fidelity one last time with our sequencing primers (Supplementary Fig. 4c).

pB7GWIWG2(I) (RNAi vector)

This RNAi vector (Supplementary Fig. 5c) was chosen because it had been successfully used for RNAi transformations of *Arabidopsis* plants in our lab. Because of this familiarity we decided to use this RNAi vector for our tomato transformation. A 300

bp segment of *Sl*APY1 (Supplementary Fig. 1c) was amplified with specific primers (Supplementary Fig. 4d) for ligation into the pENTR-D/TOPO vector. Successful ligation was screened for by PCR using the M13(+) primer. One of these constructs was then chosen for sequencing using the forward and reverse M13 primers. Once fidelity was assessed the construct was then transformed into *E. coli* so that isolated plasmids could be collected. These constructs were next used as entry vectors for the LR recombination with pB7GWIWG2(I) by LR clonase. The recombined constructs were transformed into *E. coli* and screened by restriction digestion. Our final RNAi vector containing a segment of *Sl*APY1 was again sequenced for fidelity.

Transformation of Tomatoes

Protocol for tomato transformation was obtained from the Gould lab. Procedures presented were modified from Park, *et. al.* (2003), and Byce, J. E. (last updated 8/1/06).

Plant Material

We sterilized seeds by immersing them in 10% Clorox plus 1 drop of detergent for 15 min. (use 90 seeds~380mg) then rinsing them well with sterile Milli-Q water (4 or more times). We sowed seeds in Petri-dishes containing 1/2 MS (Murashige & Skoog, 1962), approximately 30 seeds/dish, and allowed them to germinate for 7 days. One day prior to inoculation with *Agrobacterium*, we cut cotyledons from 6 – 8-day-old seedlings, placed them on Medium 1 [MS+1mg/L BAP + 0.1mg/L NAA], and cultured them for 1 day in light at room temperature.

Agrobacterium

We grew colony(s) of *Agrobacterium* tumefaciens EHA105 containing Apyrase overexpression or knockdown constructs, in 5-6 ml liquid LB media containing 100 mg/L kanamycin, rotated at 200 rpm overnight at 25-26°C. Do not exceed 27°C, because the Agro transformation process is temperature sensitive. The following day, we centrifuged the cell suspension, decanted the supernatant, and suspended the pellet in 5-6 ml of Virulence Induction Medium (VIM). [VIM (glucose 20 g/l + AS 20 mg/L + MES 75 mM pH 5.4]

Inoculation & Co-cultivation, Selection, and Rooting

We incubated cotyledon explants with *Agrobacterium* suspended in VIM ~15-20 min. The inoculated explants were then placed on Medium 1 plus 20mg/L Acetoysyringone (AS) in the dark at 20°C for 3 days. [MS+BAP 1mg/L + NAA 0.1 mg/L + AS 20~60mg/L]. After 3 days, explants were transferred to Media 2 [MS + Zeatin 2mg/L + IAA 0.1mg/L + Clavamox 250mg/L + kanamycin 100~150mg/L] where they were cultured at room temperature with a 16-hr photoperiod. Explants were transferred to fresh Medium 3 with Clavamox and kanamycin every 2-3 weeks. We observed shoots appearing within 4 - 6 weeks. Shoots were excised from explants when shoots were about 2 cm tall. They were placed in large culture containers (Kerr or Ball half-pint canning jars, baby food jars, Magenta Boxes, deli containers, etc.) containing Rooting Media with a selective agent and antibiotic used to prevent *Agrobacterium* growth. [RM+ Selection: MS + Clavamox 250mg/l + Kanamycin 150mg/l].

IV. Results

Sequence

Solanum lycopersicum, MicroTom was the tomato cultivar used to determine the apyrase sequence. No known full cDNA sequence of *Solanum lycopersicum* apyrase has been identified. Therefore based on the *Arabidopsis* apyrase amino acid sequence we created a putative *tomato* apyrase cDNA sequence (Supplemental Fig. 1a) from *Solanum lycopersicum* expressed sequence tags (EST). When our final sequence underwent a nucleotide BLAST against tomato EST, we found multiple sequences with alignment scores above 200. This sequence was amplified from a Micro-Tom leaf cDNA library.

Additionally an amino acid alignment of the *Solanum lycopersicum* apyrase sequence (Supplemental Fig. 1b) with *Arabidopsis* apyrase 1 (*At*APY1) resulted in a 49% identity and 82% similarity (Fig. 1a). While the amino acid alignment with apyrase 2 (*At*APY2) resulted in a 51% identity and 82% similarity (Fig. 1b). Alignments were made with the sequence analysis tool, ClustalW2 from the European Bioinformatics Institute (EBI) website. Due to the tomato apyrase amino acid sequence having roughly 80% similarity to both *At*APY1 and *At*APY2, we believe that it is appropriate to identify this sequence as *Solanum lycopersicum* apyrase 1 or *Sl*APY1. No other putative *Solanum* apyrase sequences were identified during our analysis.

Using the amino acid sequence of *Sl*APY1 to predict transmembrane domains, we discovered that tomato apyrase *Sl*APY1 is most likely an ectoapyrase with one membrane spanning domain. The program used was TMHMM which has an N-best algorithm. The

results (Fig. 2) show a roughly 20 amino acid (#7-27) transmembrane helix on the N-terminus. This region is speculated to be a transmembrane domain but could also be a signal peptide sequence. Concurrently when *At*APY1 and *At*APY2 are analyzed with the TMHMM program both resulted in one transmembrane domain (Supplementary Fig. 3a and Fig. 3b respectively). Interestingly, *At*APY1 also has the transmembrane domain in the first 60 amino acids of the N-terminus.

Differential expression of *Sl*APY1 in various tomato tissues

First strand cDNA libraries were made from a variety of tissues from the wild-type Micro-Tom tomato. The following tissues were collected: red fruit (mature), red fruit skin, green fruit (immature), flower, and young leaves. Internal *Sl*APY1 primers (Supplementary Fig. 4a) were used to assess the relative concentration of *Sl*APY1 mRNA in the various tissues. RT-PCR was performed with NEB Quick-Load Taq 2X master mix with an annealing temperature of 52 °C. The number of cycles (30) was adjusted so that visible differences between band intensity were seen. Our results indicate that *Sl*APY1 expression is the highest in young leaves, then expressed less in the green fruit, and expressed the least in the flower (Fig. 4). Expression in red fruit and the skin of the red fruit was not seen after 30 cycles. However the results of our experiment are inconclusive. The RNA isolation of certain samples (red fruit and red fruit skin) did not yield sufficient RNA concentrations needed to make uniform cDNA libraries, thus a loading control needs to be run to confirm results.

Transformed tomatoes overexpressing a tomato ectoapyrase, *Sl*APY1

Potential transgenic plants were sent to the Roux lab for screening after *Agrobacterium* mediated transformation of Micro Tom tomatoes with the *Sl*APY1 overexpressor vector and pCNL56 control vector. Plants were received in sterile containers with callus growth. Due to this vulnerable stage of plant growth, transgenic plants were allowed to grow for an additional 1 ½ months. Once plants had normal phenotypes they were transplanted into soil. During this time, we had five healthy transgenic plants potentially overexpressing *Sl*APY1, and over 10 transgenic plants with the control vector (confirmed by a GUS stain). Two weeks after transplantation, young leaves were collected for first strand cDNA synthesis. Internal *Sl*APY1 primers (Supplementary Fig. 4a) were used for RT-PCR. Extra care was taken to make sure the same amount of RNA was used to create each cDNA library. The resulting PCR revealed two lines (21 2(1) and 21 (1)) that are highly likely overexpressing *Sl*APY1 (Fig. 3). A loading control is needed to confirm these results. These two lines are the F1 generation, thus they have not been further analyzed for phenotypic differences. Currently seeds are being collected so that other observations can be made with the F2 generation.

V. Discussion

Due to the high percent similarity between *Sl*APY1 and *At*APY1 and *At*APY2, we believe the functions of these proteins could be related. Additionally the prediction of a transmembrane helix (TMH) in *Sl*APY1 is further indication that *Sl*APY1 is similar to the ectoapyrases *At*APY1 and *At*APY2 which both have one TMH. However the location of *Sl*APY1's TMH is in the first 60 amino acids of the protein, which could imply this region is instead a signal peptide. Nevertheless, we still believe *Sl*APY1 is likely an ectoapyrase due to the fact that *At*APY1, a confirmed ectoapyrase whose percent similarity with *Sl*APY1 is 82 %, also has its TMH in the N terminal region of the protein.

With the likely chance that *Sl*APY1 is related to *Arabidopsis* (*At*) APY1 and APY2, the growth regulatory ability of these *Arabidopsis* ectoapyrases could be a possible function of *Sl*APY1. Thus to further elucidate the function of *Arabidopsis* ectoapyrase we decided to perform a few experiments that chemically inhibit root hair growth. To do this we first used a known apyrase inhibitor NGXT191 to see if *Arabidopsis* root hair growth could be inhibited. Using data provided by Windsor, B (2000) PhD dissertation, The University of Texas at Austin, we already knew that NGXT191 has no inhibitory effect on acid phosphatase. But instead has almost equal inhibitory activity for apyrase and alkaline phosphatase. Thus to ensure that alkaline phosphatase is not the enzyme regulating eATP concentrations we performed a control experiment where 500 μM levamisole (a known alkaline phosphatase inhibitor) was applied to root hairs. Our results indicated that when levamisole was applied, root hair

growth did not change, however when an apyrase inhibitor (NGXT191) was applied, inhibition of root hair growth was observed (data provided in the appendix). Thus we believe apyrase is the key enzyme in limiting eATP concentrations to below inhibitory levels. These inhibitory levels can be artificially created to observe root hair inhibition by application of ATPγS, a poorly-hydrolysable form of ATP. This form of ATP was chosen because of its relative resistance to hydrolysis by ATPases, thus eliminating or greatly reducing the likelihood that it could be used as an energy source or as a source of a phosphate group during phosphorylation. From our results we consistently saw inhibition of root hair growth when 125 μM ATPγS or more was applied to root hairs (data provided in the appendix). These two experiments further indicate *Arabidopsis* ectoapyrases' role in growth regulation through control of eATP concentrations.

A preliminary experiment of applying 150 μM ATPγS to tomato root hairs using the same method as the *Arabidopsis* root hair experiments (see Appendix) resulted in a significant inhibition of tomato root hair growth in one hour (Supplementary Fig. 6). Only one trial was performed due to the constraints of measuring tomato root hairs which are extremely short and densely packed. However this preliminary experiment revealed to us the high possibility that *Sl*APY1 and *At*APY1 and *At*APY2 have the same function of regulating eATP concentrations as a proxy to controlling growth.

In addition to uncovering the probable function of *Sl*APY1 we were also interested in the expression profile of *Sl*APY1 in wild-type Micro-Tom tomatoes. The tissues we collected included young leaves, flowers, green (immature) fruit tissue, red (mature) fruit tissue, and red fruit skin. We were unable to collect green fruit skin due to

the attachment the skin had to the fruit, making it almost impossible to cleanly remove a large portion of the skin. From our results we found *Sl*APY1 to be highly expressed in young leaves. More surprisingly was the expression of *Sl*APY1 in the green fruit but not in the red fruit of tomatoes. This finding is promising since it shows that this tomato apyrase is expressed during early stages of fruit development where the most growth occurs. Additionally, when growth stops and fruit ripening begins, apyrase expression disappears. These findings still need to be verified with an appropriate loading control. However, if our results are correct our assumption that overexpressing *Sl*APY1 will affect fruit growth is strengthened due to the localization of *Sl*APY1 expression in green fruit.

Once the sequence for *Sl*APY1 was established, we began to clone this gene to be constitutively expressed by a CaMV 35S promoter. During this process we revised the cloning strategy three times until a successful construct was produced. The overview of the final strategy is outlined in the materials and methods section. However to summarize the lessons I learned during my thesis it will be helpful to run through the various permutations my strategy had. My first strategy failed due to my oversight of an ATG start codon present in the restriction site I added to the *Sl*APY1 sequence. This mistake was not discovered until after I had cloned this insert into pRT100 and had sequenced the construct. Due to my oversight I carefully re-analyzed my strategy and decided to choose two different restriction sites with compatible restriction digest conditions. Unfortunately one of these RE sites created blunt ends after digest. I soon discovered how tenuous blunt end ligation could be and after several failed attempts I

returned to my strategy for revision. This time I had only one more option left due to the limited RE sites present in pRT100. My final strategy was to mix and match my previous strategies and use the C terminal RE site I had in my first strategy and the N terminal RE site I had in my second strategy, both producing sticky ends. The only problem I had was that these REs did not have compatible digest conditions. Thus I had to be extremely careful in keeping my yields high even after a serial digestion. In addition to this major revision I also decided to first clone my *Sl*APY1 sequence into a TOPO vector so that I could assess fidelity before I continued any further in my project. With these changes I was soon able to successfully clone *Sl*APY1 into pRT100 and then clone *Sl*APY1 with its appropriate promoter and terminator regions into pCAMBIA 2300 for transformation. Due to my previous errors in cloning I made sure I did not repeat any mistakes when designing my RNAi strategy. Because of these lessons learned, I quickly and successfully cloned a portion of the *Sl*APY1 sequence into the RNAi vector.

Once these constructs were made we sent them to the Gould lab at Texas A&M University to be transformed into Micro-Tom tomatoes. Once the transformation process was completed we received several plants that contained the control vector, pCNL56, confirmed by a GUS stain (example in Supplementary Fig. 7). Additionally we received potential transgenic tomato plants that overexpress *Sl*APY1. Through RT-PCR screens we believe that two of the seven lines are overexpressing *Sl*APY1 when compared to transgenic tomato plants with the control vector. Unfortunately due to the extensive time spent cloning two vectors and the relatively long generation time of Micro-Tom tomatoes, we were unable to perform any further experiments. Ideally we would have

liked to run a loading control for our RT-PCR screen with either tomato actin or ubiquitn primers. Even more helpful would have been a western blot to analyze the protein concentrations of the various lines. This experiment would be problematic due to no known antibody for *Sl*APY1. However we were hoping to use *At*APY1 or *At*APY2 antibodies for a western blot hoping that the high percent similarity between these proteins would result in *At*APY1 and *At*APY2 antibody recognition for *Sl*APY1.

If these lines are indeed overexpressing *Sl*APY1 we would be most interested in observing the phenotype of these transgenic plants. Apyrase expression has been found to be localized in tomato skin and in guard cells. If *Sl*APY1 is overexpressed in these tissues we would expect to see some type of phenotype change, whether it is increased fruit size or even increased stomata opening. These potential changes could have a huge impact on our understanding of the role *Sl*APY1 has in fruit growth and even in drought resistance.

Figures

(A.)

```
SlAPY1          ----------------MQKHN-----ISNVYNLFNIMLLILVGLPLSSHANDYSEK---- 35
AtAPY1          MTAKRAIGRHESLADKVHRHRGLLLVISIPIVLIALVLLLMPGTSTSVSVIEYTMKNHEG 60
                               :::*.     **    *: ::**:: * . *  . :*: *

SlAPY1          --------KYAVIFDAGSTGSRVHVFRFNSNLDLINIGNDLELFLQIKPGLSSYADDPKA 87
AtAPY1          GSNSRGPKNYAVIFDAGSSGSRVHVYCFDQNLDLVPLENELELFLQLKPGLSAYPNDPRQ 120
                        :*********:******: *:.****: : *:******:*****:*.:**:

SlAPY1          AANSLKPLLEKAEAVIPKNLQSQTPIKVGATAGLRLLKGDSSEKILQAVRDMLKNETTLS 147
AtAPY1          SANSLVTLLDKAEASVPRELRPKTPVRVGATAGLRALGHQASENILQAVRELLKGRSRLK 180
                :**** .**:**** :*::*:.:**::******** *  ::**:******::**..: *.

SlAPY1          YKDEWVSVLEGTLEGSYFWVSLNYLYGNLGKNYPDTIATIDLGGGSVQIAYAVSKQSAIN 207
AtAPY1          TEANAVTVLDGTQEGSYQWVTINYLLRTLGKPYSDTVGVVDLGGGSVQMAYAIPEEDAAT 240
                 : : *:**:** **** **::***  .*** *.**:..:********:***:.::.* .

SlAPY1          APKLPNG-DAYVQQKALLGTNYYLYVHSFLNYGLLAARADILKASKNYTSPCIVEGHNGT 266
AtAPY1          APKPVEGEDSYVREMYLKGRKYFLYVHSYLHYGLLAARAEILKVSEDSNNPCIATGYAGT 300
                ***  :* *:**::  * * :*:*****:*:********:***.*:: ..***. *: **

SlAPY1          YTYNGVSYKAASRKQGPNIRRCKAIIRKLLQID-APCNHKNCSFAGIWNGGGGAGTKNLY 325
AtAPY1          YKYGGKAFKAAASPSGASLDECRRVAINALKVNNSLCTHMKCTFGGVWNGGGGGGQKKMF 360
                *.*.* ::***:  .*..: .*: :  : *::: : *.* :*:*.*:******.* *:::

SlAPY1          ISSFFYDYASTVGIVDPKEAYGITQPIQYYKAATLACKTKKQNMKSVFPNINDKDIPFIC 385
AtAPY1          VASFFFDRAAEAGFVDPNQPVAEVRPLDFEKAANKACNMRMEEGKSKFPRVEEDNLPYLC 420
                ::***:* *: .*:***::. . .:*::: ***. **: : :: ** **.:::.::*::*

SlAPY1          MDLLYEYTLLVNGFGIDPIRKITVVHQVNYKNHLVEAAWPLGSAIDAVSSTTSENMISYV 445
AtAPY1          LDLVYQYTLLVDGFGLKPSQTITLVKKVKYGDYAVEAAWPLGSAIEAVSSP--------- 471
                :**:*:*****:***:.* :.**:*::*:* :: ***********:****.

SlAPY1          GRISY 450
AtAPY1          ----- 
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(B.)

```
SlAPY1          ------------------------------------------------------------
AtAPY2          MLNIVGSYPSPAIVTHNVFCLHPSLSHTKFRSEAHTSFGFQIKSGDSSRFPKFTVDLEPL 60

SlAPY1          ------------------------------------------------------------
AtAPY2          QDPPQTTASSGTGNGNGKIRYRSPSSTELLESGNHSPTSDSVDGGKMTAKRGIGRHESLA 120

SlAPY1          --MQKHN-----ISNVYNLFNIMLLILVGLPLS-SHANDYSE------------KKYAVI 40
AtAPY2          DKIQRHRGIILVISVPIVLIGLVLLLMPGRSISDSVVEEYSVHNRKGGPNSRGPKNYAVI 180
                  :*:*.     **    *:.::**:: * .:* * .::**             *:****

SlAPY1          FDAGSTGSRVHVFRFNSNLDLINIGNDLELFLQ--------------------------- 73
AtAPY2          FDAGSSGSRVHVYCFDQNLDLIPLGNELELFLQSLVKKLASPNGSNRANMTLFDHGNISC 240
                *****:******: *:.***** :**:******

SlAPY1          ------------------------IKPGLSSYADDPKAAANSLKPLLEKAEAVIPKNLQ 108
AtAPY2          PEVKLNRINGKLRTLLSMYIIDLCSLKPGLSAYPTDPRQAANSLVSLLDKAEASVPRELR 300
                                       :*****:*. **: ***** .**:**** :*::*:

SlAPY1          SQTPIKVGATAGLRLLKGDSSEKILQAVRDMLKNETTLSYKDEWVSVLEGTLEGSYFWVS 168
```

```
AtAPY2          PKTHVRVGATAGLRTLGHDASENILQAVRELLRDRSMLKTEANAVTVLDGTQEGSYQWVT 360
                .:* ::******** *  *:**:******::*::.: *. : : *:**:** **** **:

SlAPY1          LNYLYGNLGKNYPDTIATIDLGGGSVQIAYAVSKQSAINAPKLPNG-DAYVQQKALLGTN 227
AtAPY2          INYLLRNLGKPYSDTVGVVDLGGGSVQMAYAISEEDAASAPKPLEGEDSYVREMYLKGRK 420
                :***  **** *.**:..:********:***:*::.* .***  :* *:**::  * * :

SlAPY1          YYLYVHSFLNYGLLAARADILKASKNYTSPCIVEGHNGTYTYNGVSYKAASRKQGPNIRR 287
AtAPY2          YFLYVHSYLHYGLLAARAEILKVSEDSENPCIVAGYDGMYKYGGKEFKAPASQSGASLDE 480
                *:*****:*:********:***.*::  .**** *::* *.*.* .:**.: :.*..: .

SlAPY1          CKAIIRKLLQI-DAPCNHKNCSFAGIWNGGGGAGTKNLYISSFFYDYASTVGIVDPKEAY 346
AtAPY2          CRRITINALKVNDTLCTHMKCTFGGVWNGGRGGGGQKNMFVASFFFDRAAEAGFVDPKQPV 540
                *: *  : *:: *: *.* :*:*.*:**** *.* **::::***:* *: .*:****:.

SlAPY1          GITQPIQYYKAATLACKTKKQNMKSVFPNINDKDIPFICMDLLYEYTLLVNGFGIDPIRK 406
AtAPY2          ATVRPMDFEKAAKKACSMKLEEGKSTFPLVEEENLPYLCMDLVYQYTLLIDGFGLEPSQT 600
                . .:*::: ***. **. * :: **.** ::::::*::****:*:****::***::* :.

SlAPY1          ITVVHQVNYKNHLVEAAWPLGSAIDAVSSTTSENMISYVGRISY 450
AtAPY2          ITLVKKVKYGDQAVEAAWPLGSAIEAVSSP-------------- 630
                **:*::*:* :: ***********:****.
```

Fig. 1 Sequence alignment of the amino acid sequence of *Sl*APY1 with **a.** *At*APY1's amino acid sequence and with **b.** *At*APY2's amino acid sequence.

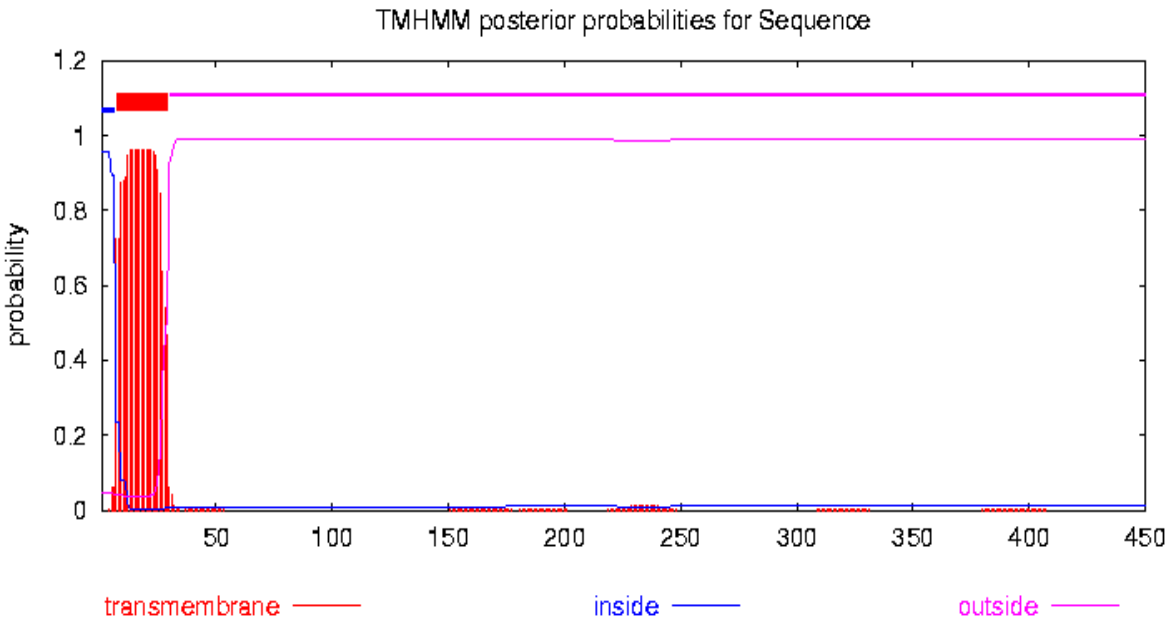


Fig. 2 Predicted transmembrane domain helix (roughly 20 amino acids) using the TMHMM program. The transmembrane domain is located in the N terminal end of the *Sl*APY1 protein.

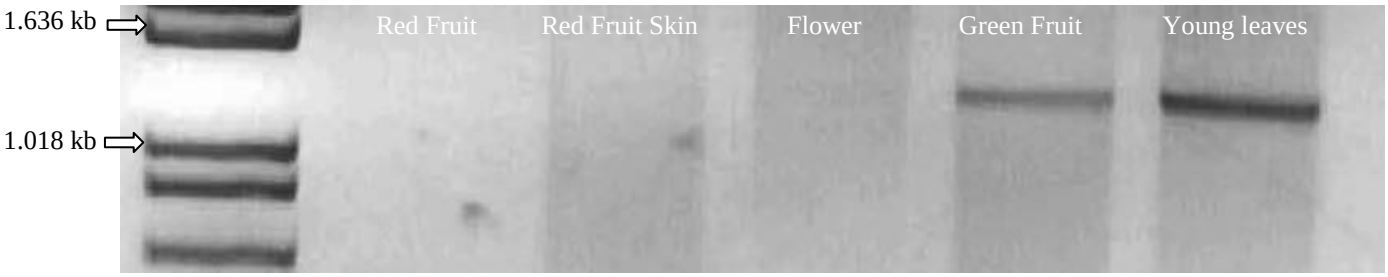


Fig. 3 RT-PCR of the differential expression of *Sl*APY1 in red fruit, red fruit skin, flower, green fruit, and young leaves.

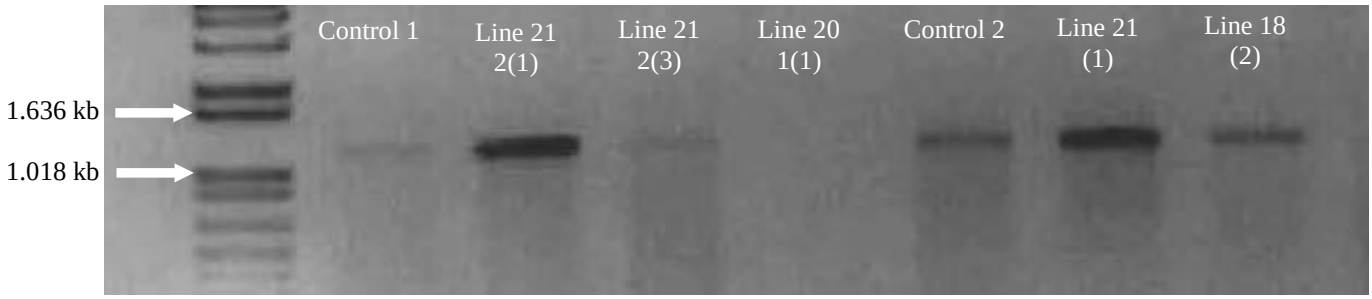


Fig. 4 RT-PCR confirmation of *Sl*APY1 overexpressing lines.

Supplemental Figures

Supplemental Fig. 1

a.) *Solanum* apyrase cDNA sequence

ATGCAGAAGCATAATATTAGTAATGTTTATAACTTGTTCAACATTATGTTGTTGATACT
TGTTGGGTTGCCATTGAGCTCGCATGCCAATGATTATTCGGAGAAAAAATATGCAGTGA
TATTTGACGCTGGAAGCACTGGTAGCAGAGTTCATGTCTTTCGTTTTAACTCAAATTTG
GATCTCATCAATATCGGCAATGATCTTGAACTCTTCTTGCAGATAAAACCAGGTCTGAG
TTCATATGCAGATGATCCAAAGGCAGCTGCAAATTCTCTAAAGCCCCTTCTTGAGAAAG
CTGAAGCTGTTATTCCTAAGAATTTACAGTCTCAAACCCCTATTAAAGTTGGGGCAACT
GCAGGGCTGAGGTTATTAAAGGGTGATTCATCTGAAAAGATTCTGCAAGCAGTAAGAGA
TATGCTGAAAAATGAAACTACTCTGAGTTACAAGGATGAATGGGTCTCTGTTCTCGAAG
GAACTCTAGAAGGTTCTTATTTTTGGGTAAGTTTGAACTATTTGTATGGGAATTTGGGC
AAAAATTACCCAGACACCATTGCTACAATTGATCTTGGAGGTGGATCAGTTCAAATTGC
TTATGCTGTCTCAAAACAAAGTGCTATAAATGCTCCAAAGTTACCAAATGGAGACGCTT
ATGTCCAACAAAAAGCACTTCTTGGAACTAATTATTACCTCTATGTTCACAGTTTTCTA
AATTATGGACTATTAGCAGCTCGAGCTGATATCTTGAAGGCTTCTAAAAATTACACTAG
TCCATGCATCGTGGAAGGGCACAATGGTACTTACACATATAATGGAGTATCTTATAAAG
CTGCATCACGAAAACAAGGTCCAAATATCAGAAGATGTAAAGCAATAATTAGAAAATTG
CTTCAGATTGATGCACCTTGCAATCACAAAAATTGTTCATTTGCTGGGATTTGGAATGG
TGGTGGTGGAGCTGGAACCAAAAATCTCTATATCTCTTCATTTTTCTATGATTATGCTT
CTACAGTTGGTATAGTGGATCCAAAAGAGGCCTATGGTATAACTCAGCCAATACAATAC
TATAAAGCAGCGACGCTGGCTTGTAAGACTAAGAAGCAAAACATGAAATCGGTATTCCC
TAACATTAACGATAAGGACATACCCTTTATCTGCATGGATTTATTATATGAATACACTT
TGCTGGTAAATGGATTTGGTATTGATCCAATAAGAAAGATTACAGTGGTGCATCAAGTT
AATTACAAAAATCACCTTGTTGAAGCTGCATGGCCATTAGGCTCTGCTATTGATGCTGT
CTCATCCACAACATCAGAAAATATGATTTCATATGTTGGGAGGATAAGTTATTAG

b.) *Solanum* apyrase amino acid sequence

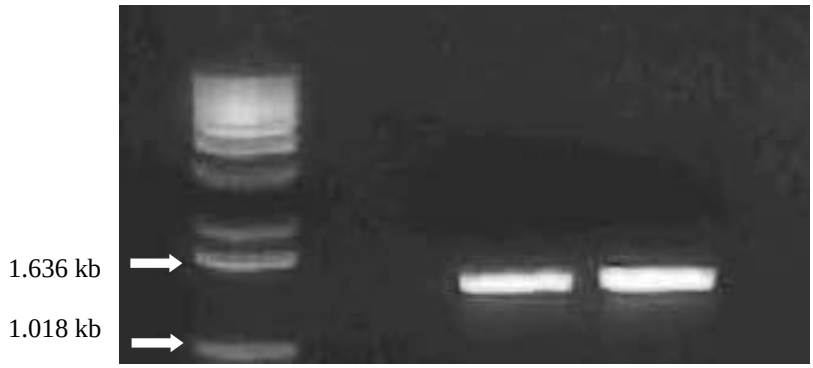
MQKHNISNVYNLFNIMLLILVGLPLSSHANDYSEKKYAVIFDAGSTGSRVHVFRFNSNL
DLINIGNDLELFLQIKPGLSSYADDPKAAANSLKPLLEKAEAVIPKNLQSQTPIKVGAT
AGLRLLKGDSSEKILQAVRDMLKNETTLSYKDEWVSVLEGTLEGSYFWVSLNYLYGNLG
KNYPDTIATIDLGGGSVQIAYAVSKQSAINAPKLPNGDAYVQQKALLGTNYYLYVHSFL
NYGLLAARADILKASKNYTSPCIVEGHNGTYTYNGVSYKAASRKQGPNIRRCKAIIRKL
LQIDAPCNHKNCSFAGIWNGGGGAGTKNLYISSFFYDYASTVGIVDPKEAYGITQPIQY
YKAATLACKTKKQNMKSVFPNINDKDIPFICMDLLYEYTLLVNGFGIDPIRKITVVHQV
NYKNHLVEAAWPLGSAIDAVSSTTSENMISYVGRISY

c.) Segment of *Sl*APY1 used for the sense and antisense portion for RNAi

AAAGTTGGGGCAACTGCAGGGCTGAGGTTATTAAAGGGTGATTCATCTGAAAAGATTCT
GCAAGCAGTAAGAGATATGCTGAAAAATGAAACTACTCTGAGTTACAAGGATGAATGGG
TCTCTGTTCTCGAAGGAACTCTAGAAGGTTCTTATTTTTGGGTTGCTTTGAACTATTTG
TATGGGAATTTGGGCAAAAATTACCCAGACACCATTGCTACAATTGATCTTGGAGGTGG
ATCAGTTCAAATTGCTTATGCTGTCTCAAAACAAAGTGCTATAAATGCTCCAAAGTTAC
CAAAT

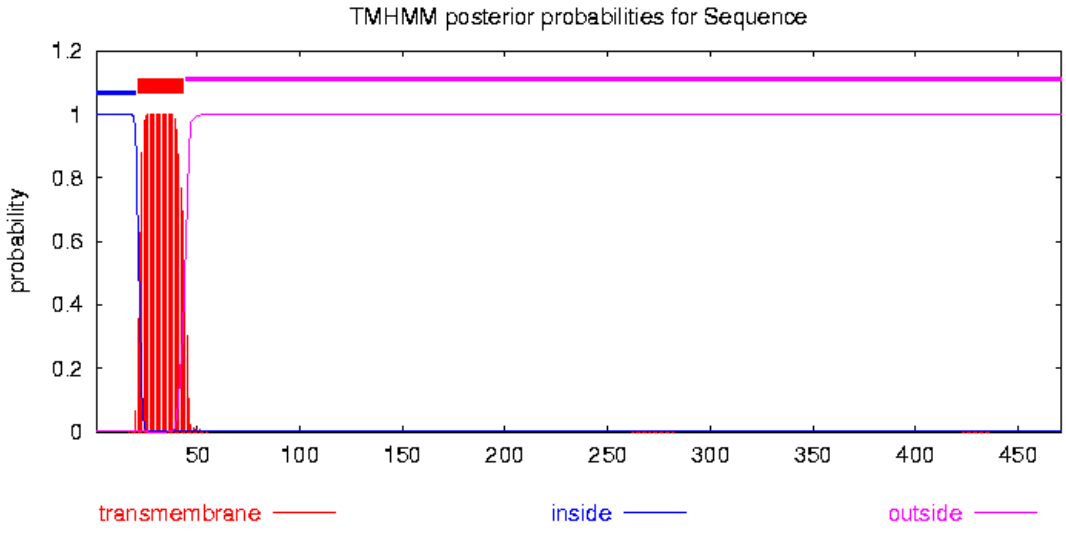
Supplementary Fig. 2

DNA gel of amplified *Sl*APY1 from Micro-Tom leaf cDNA, size – 1.3 kb

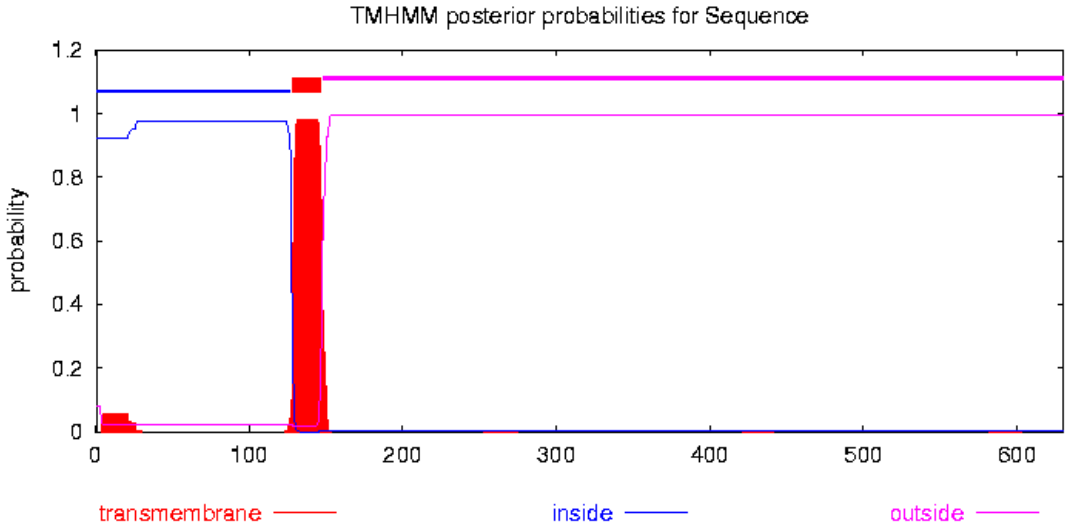


Supplementary Fig. 3

a.) Predicted TMH for *At*APY1



b.) Predicted TMH for *At*APY2



Supplementary Fig. 4

a.) *Internal SlAPY1 primers*

Forward 5' – AAAAAATATGCAGTGATATTTGAC

Reverse 3' – GGATGAGACAGCATCAATAGCAGA

b.) *SlAPY1 primers with specified restriction enzyme cut site*

Forward 5' – GCCGGGGCCCATGCAGAAGCATAATATTAGTAAT (with ApaI site)

Reverse 3' – CTAGGGTACCCTAATAACTTATCCTCCCAACATA (with KpnI site)

c.) *Sequencing primers using 35S promoter and terminator regions*

Forward 5' – ATGCCTCTACCGACAGTGGTC

Reverse 3' – ATTTGTAGAGAGAGACTGGTGAT

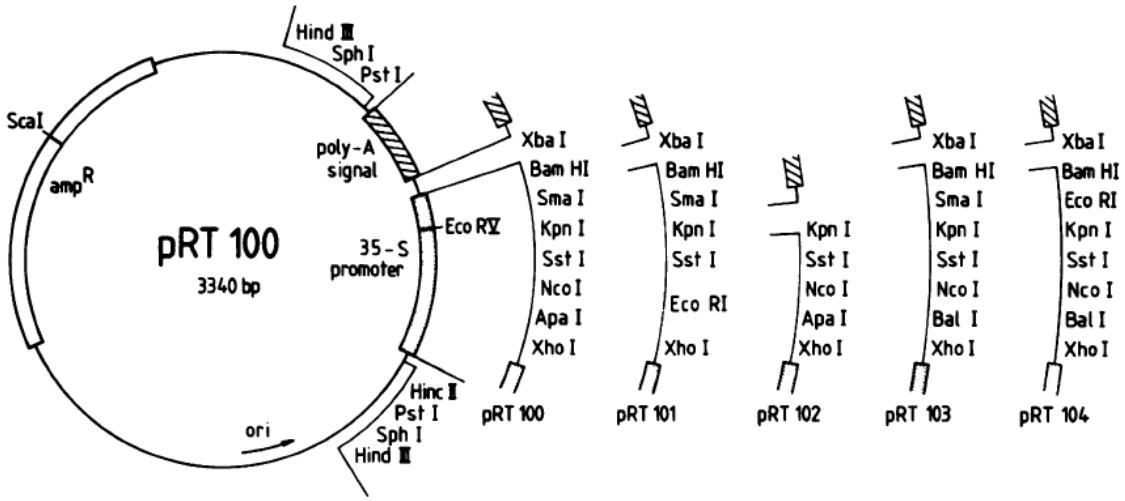
d.) *RNAi primers*

Forward 5' – CACCAAAGTTGGGGCAACTGCAGG

Reverse 3' – ATTTGGTAACTTTGGAGCATTTAT

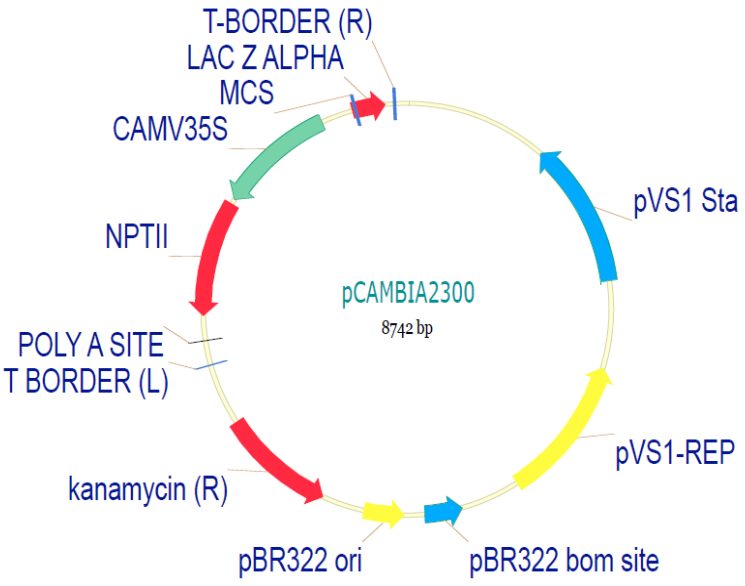
Supplementary Fig. 5

a.) pRT100 vector

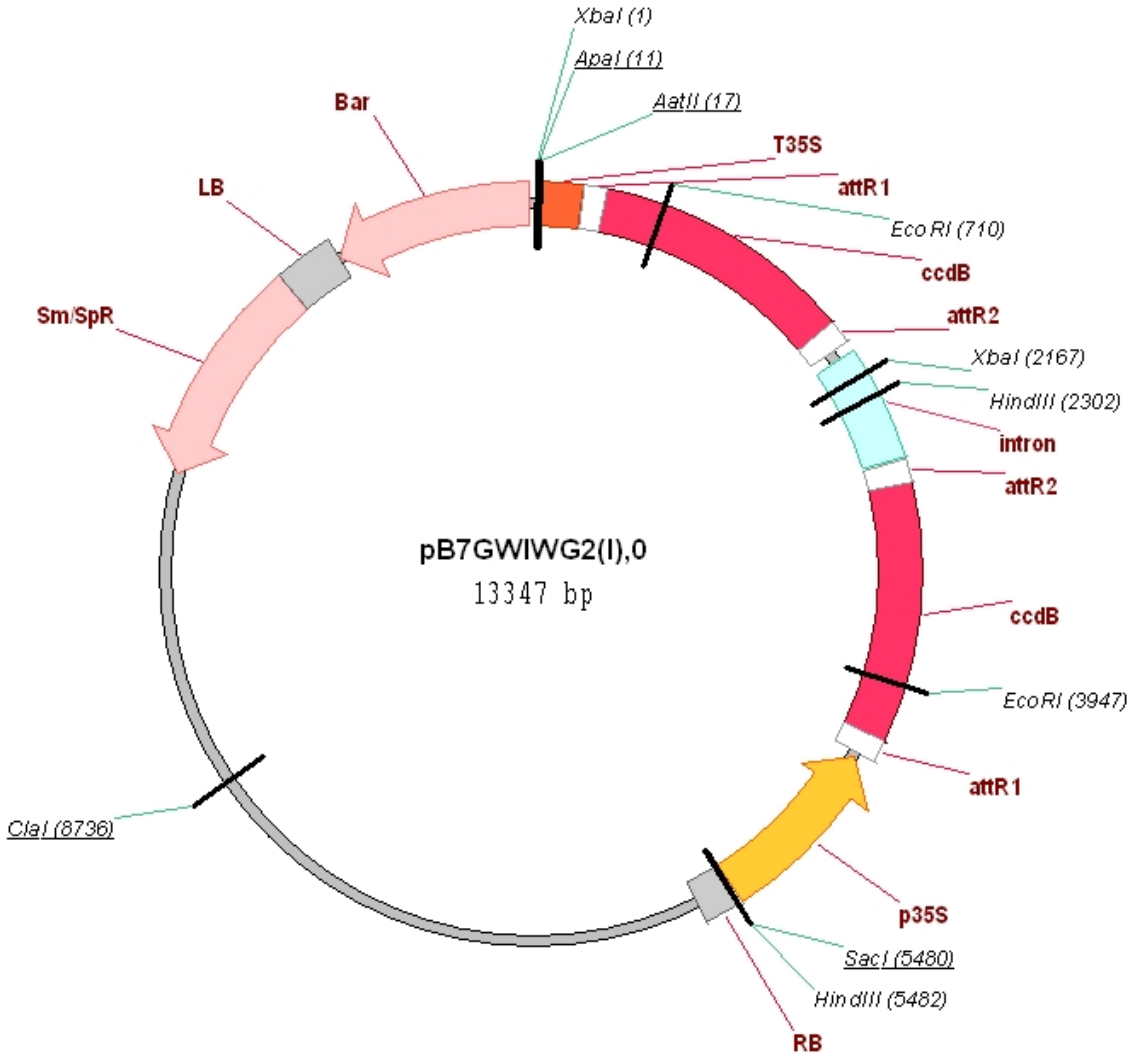



```
Hind3 SphI  PstI  HincII
aagcttgcatgcctgcaggtcAACATGGTGGAGCACGACACTCTCGTCTACTCCAAGAATATCAAAGATACAGTCTCAGAAGACCAGAGGGCTATTGAGACTTTTCAACAAAGGGTAATA 120
TCGGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCGAAAGGACAGTAGAAAAGGAAGATGGCTTCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAA 240
                                                                                                    EcoRV
GAATGCCTCTACCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAACATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA 360
                                                                  XhoI ApaINcoI    SstI  KpnISmaI BamHI
CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACctcgagggcccatgggcgagctcggtacccggggatcct 480
XbaI
ctagagtcGCGAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTATTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCTTATAGGGTTTCGCTCATGT 600
                                                                                  PstI  SphI  Hind3
GTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAATTTCTAATTCCTAAAACCAAAATCCAGTgacctgcaggcatgcaagctt 711
```

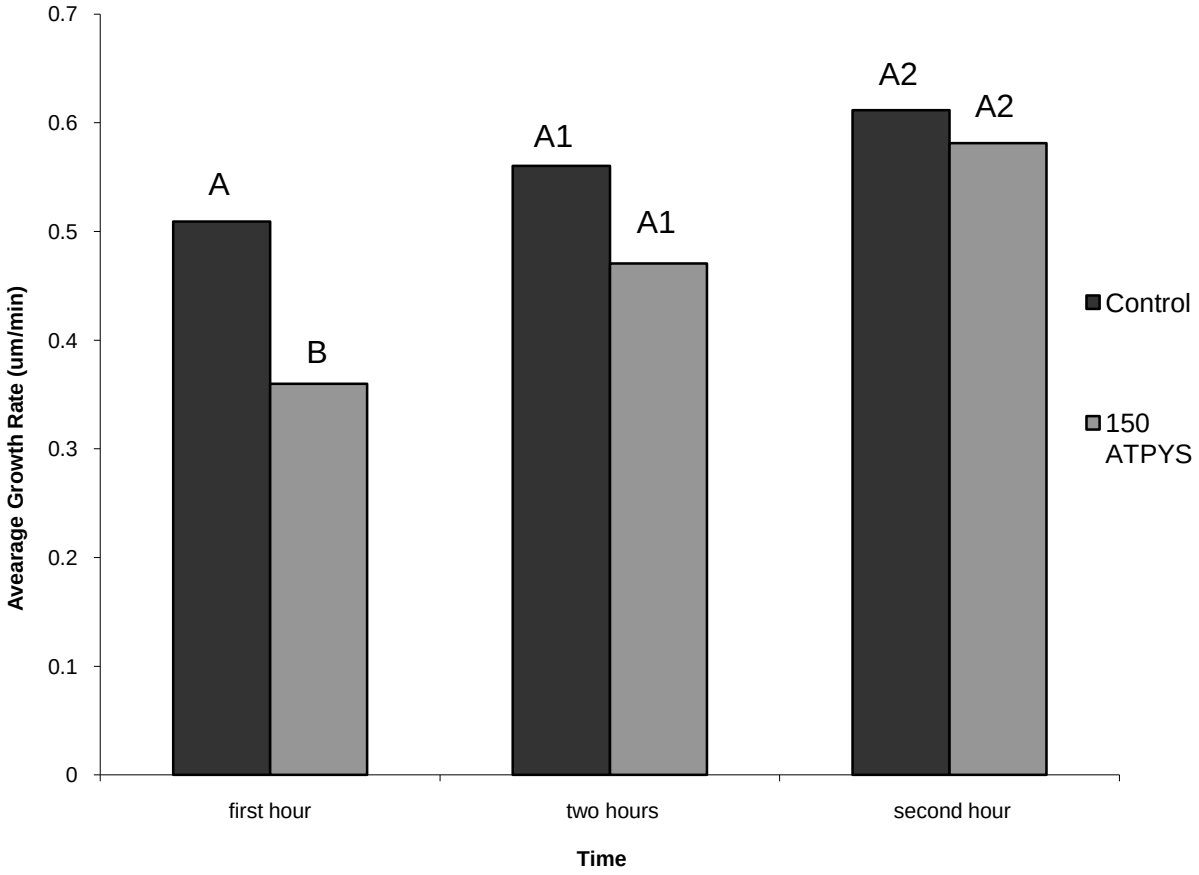
b.) pCAMBIA 2300 vector



c.) pB7GWIWG2(I) vector

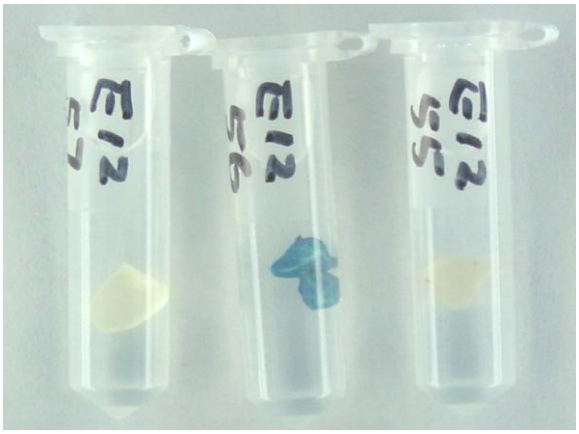


Supplementary Fig. 6 Tomato root hair experiment with application of high ATPγS



Supplementary Fig. 7

Gus staining confirming the presence of the control vector, pCNL56, in transformed tomatoes. Work done by the Gould lab at Texas A&M University.



X. Appendix:

Arabidopsis material and growth conditions

Arabidopsis thaliana ecotypes Columbia were used as the wild type in our study. Seeds were first surface sterilized in 20% (v/v) bleach for 10 minutes and then rinsed with autoclaved water five to seven times. The sterilized seeds were allowed to vernalize in 4°C for at least three days. Prepared seeds were next planted directly on a cellophane membrane placed upon solidified Murashige and Skoog (MS) medium (4.3g/L MS salts (Sigma), 0.5 % (w/v) MES, 1% (w/v) sucrose, and 1.0% (w/v) agar, raised to pH 5.7 with 1 M KOH). Planted plates were placed upright in a culture chamber and grown at 23° C under 24-h fluorescent light for 3 ½ days.

Root Hair Experiments

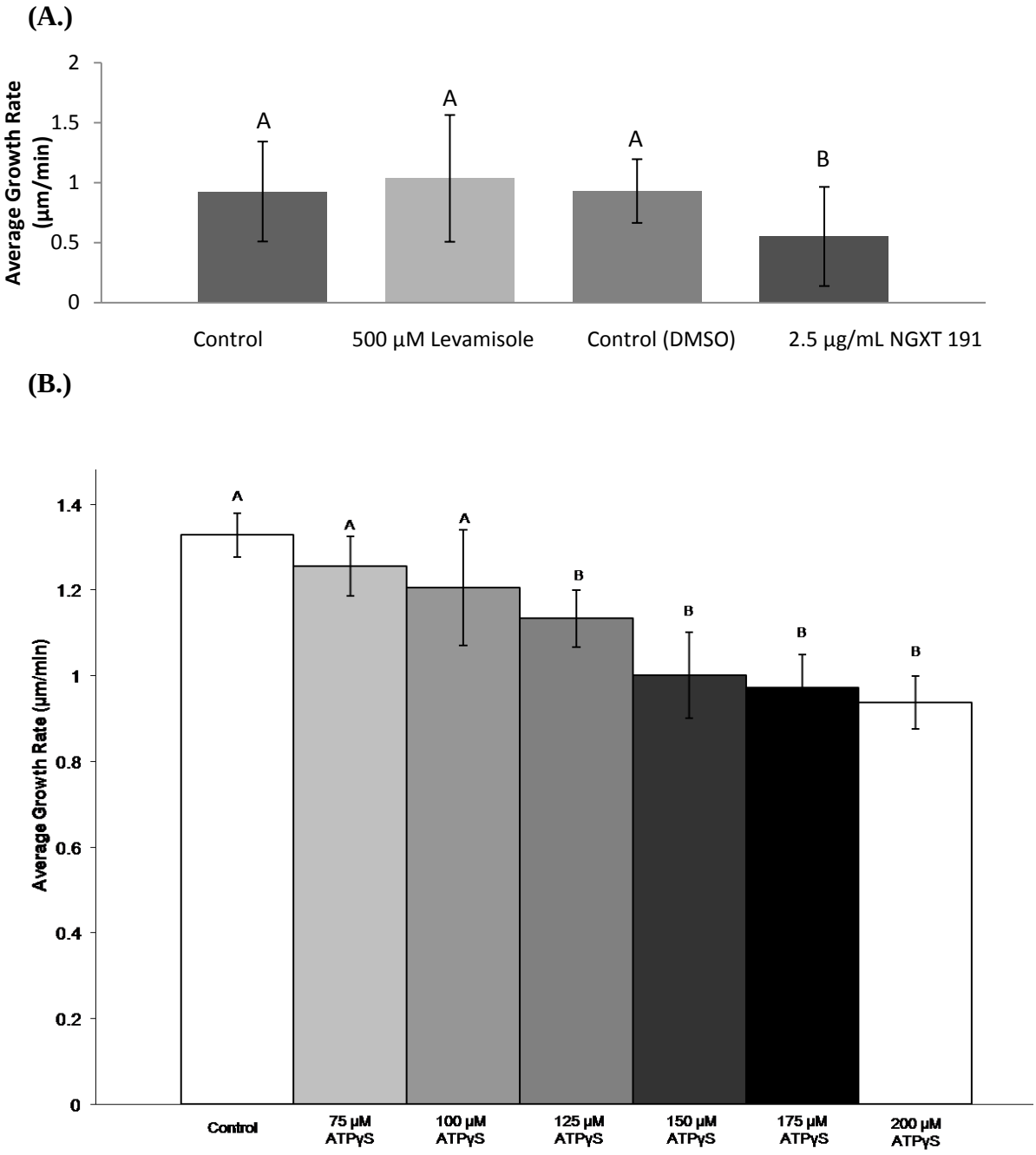
Seeds used for microscopic analysis were grown in the same environmental conditions on cellophane and MS agar plates. After 3 ½ days of growth, plates were prepared for transfer. Experimental plates were made the day of the experiment with the same agar composition used for planting. Each experimental plate had a designated chemical concentration that was added to the agar before solidification. Transfers of the seedlings were performed by lifting the cellophane membrane with tweezers from the original plate to the solidified experimental plates. Air bubbles were removed with tweezers by gently guiding the bubbles to the edge of the cellophane. Pictures of root tips were taken immediately after transfer using Motic Images Plus 2.0 under 40X

magnification at time 0 and time 60. Analysis of the root hair growth for the hour period was performed using ImageJ. Root hairs at time 0 with lengths greater than 150 μm were not used.

ATPγS and levamisole stocks were made in purified H2O while the NGXT 191 stock was dissolved in DMSO. Stocks made of DMSO were made so that when added to the MS agar plates they had a final concentration of 0.1% DMSO. Additionally when chemicals were made with DMSO, the control plates were made with the same final concentration of 0.1% DMSO that was in the treatment plate.

Chemical approaches to inhibit root hair growth

Portions of the Clark *et. al.*, in review manuscript relate apyrase function to extracellular ATP signaling. In theory ectoapyrases are present in the plasma membrane of the plant cell to regulate eATP concentrations. Ectoapyrase *At*Apy1 and *At*Apy2 have been found to be expressed in *Arabidopsis* root hairs (Wolf *et. al.*, 2007). Therefore when 2.5 μM apyrase inhibitors are applied exogenously to root hairs, a significant decrease in root hair growth rate is observed (Fig. Appendix 1a). Additionally the application of roughly 125– 150 μM ATPγS, a non-hydrolyzable form of ATP, can cause significant decreases in root hair growth after one hour (Fig. Appendix 1b).



Appendix Fig. 1 Chemical inhibition of root hair growth caused by **a.** NGXT191 an apyrase inhibitor and **b.** exogenous ATPγS. Different letters above the bars indicate significant differences between treatments ($p < 0.05$; $n \geq 30$). These results represent three biological repeats.

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X. Acknowledgements

As most of you know the Roux lab is my unofficial second home, I often feel like I spend more time in the basement of the BIO building than at my own small one bedroom efficiency. But because of my frequent visits to the lab I have had the opportunity to work on two projects, mentor three amazing generations of FRI students, and build and develop great friendships. Because of these reasons saying "bye, bye, bye" is that much harder ('N sync's Bye Bye Bye, 2000).

I would like to first thank Dr. Roux for sparking my interest in plant biology and research in general. His enthusiasm for research is unsurpassable and can only be compared to a love a fat kid has for cake (50 cent's "21 Questions", 2003). It has been my privilege to not only work in Dr. Roux's lab but also sit in his classroom. Because of his class, I now have a new found appreciation and love for the plant world.

I would also like to thank Dr. Clark for four years of collaboration and encouragement. If it wasn't for him I would have graduated college without any research experience. But because of Dr. Clark's guidance and endless opportunities for free food I have "made it through the wilderness" of college and research (Madonna's Like a Virgin, 1990).

To all my other lab mates, Araceli Cantero, Sonya Chiu, Devin Fraley, Elizabeth Henaff, Julia Kays, Stuart Reichler, Jim Tseng, Jian Wu, and Jian Yang, thank you for answering the multitude of questions I always seem to have. I would like to personally thank Elizabeth Henaff and Jian Wu for being incredibly helpful TA's during my FRI years and continuing their goodwill even after I completed the program. Sonya and Jim,

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I would like to say once again, “Thanks for the memories, thanks for the memories”, from spontaneous snowman building, to Trudy lab lunches, to Dripping Spring’s barbeque potluck, to the FRI summer of 2007 and 2009, to FRI dinners/karaoke, to the coffee or cocktail table debate, or to simply lunch time banter (Fall out boy’s Thnks fr th mmrs, 2007). “In my life”, I will never forget my time in the Roux lab. Even if “these memories lose their meaning, I’ll never lose affection” for the people that made this experience possible (Beatle’s In my life, 1965). Thank you, Roux lab.